Attempt to express the *Xerophyta viscosa* stress-responsive gene, *Xvcor*, in yeast with view to functional analysis

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Abstract

Low temperature is one of the environmental factors that cause substantial crop losses in the world. Recent advances in the study of plants native from temperate regions, have established that cold acclimation is regulated at the gene expression level. To study the function of a *Xerophyta viscosa* cold responsive protein that accumulates in plant cells under various environmental stresses, we have cloned the *Xerophyta viscosa* cold-responsive cDNA gene designed *Xvcor,* into a less complex host, *Saccharomyces cerevisiae* with the purpose of carrying out *in vivo* functional analysis. Sequence analysis showed that *Xvcor* encodes a 264 amino acid residue protein (Garwe *et al* 2002). The Hydropathy plot indicated that the XVCOR protein is highly hydrophobic and contains 6 transmembrane domains (Garwe *et al* 2002*).* In order to achieve high-level expression of *Xvcor*, the gene was placed under the control of a strong promoter (Phosphoglycerate kinase). Reverse-transcription PCR amplification revealed that the *Xvcor* transcript accumulated in yeast cells. However, SDS-PAGE analysis could not detect the predicted 29.6 kDa recombinant protein, suggesting that the translation might be hampered. Tests designed to measure the enhancement of stress tolerance between the recombinant and the control showed similar growth performance, confirming the absence of recombinant protein. A computer search for codon usage showed that the codon usage bias in *Xvcor* was low compared with that of highly expressed genes of *S. cerevisiae.* Together, these results suggest that the codon usage in *Xvcor* could influence its expression in yeast.

Introduction

Low temperatures influence the geographic distribution of plants around the world, and cause substantial economic and crop losses. However the developmental cycle of many plants from temperate regions demonstrates that they have developed the ability to cold acclimate upon exposure to low but non-freezing temperature (Thomashow, 1999). This trait is characterised at the cellular level by physiological, metabolic and genetic alterations. These changes generally include increased levels of sugars, proteins, proline, quaternary amines, and the appearance of new enzymes (Strand *et al.*, 1999). Investigations have indicated that the plasma membrane plays a crucial role in the process of freezing tolerance (Levitt, 1980). Early works have shown that remarkable changes in lipid and proteins compositions occur to increase plasma membrane fluidity during cold acclimation. Therefore we can assume that most of the molecules synthesised during cold acclimation act to stabilise the plasma membrane against freezing injury by a mechanism not yet elucidated. For example studies with Cowpea indicated that dehydrins that belong to a family of proteins, LEA-like protein D11, protect the plasma membrane under low-temperature and drought (Ismail *et al.*, 1999). But the mechanism whereby they act is poorly understood. However studies have highlighted some common features that stress encoded proteins share: they are highly hydrophobic and remain soluble upon boiling; many comprise simple chain of amino acids residues and form amphipathic alpha-helices (Thomashow, 1999).

Because changes in gene expression are considered as the upstream event triggering metabolic and physiological adaptations during cold acclimation, the new approach to address the cold stress problem has been to isolate and characterise genes that play an

important role in cold acclimation (Thomashow, 1999). The ultimate goal is to bioengineer crops for improved freezing tolerance. In order to achieve this, many *COR* (Cold Responsive) genes have been isolated from the model plant *Arabidopsis thaliana* (Thomashow, 1998) and the function of the encoded protein determined. *COR15a* for example is presumed to stabilise membranes by decreasing the propensity of membranes to form hexagonal II phase lipids in response to freezing (Artus *et al.*, 1996). Identical studies in alfalfa, wheat and barley revealed cold up-regulated genes with hypothetical roles in freezing tolerance: *(cas15)*, *(wcs120)* and *(HVA1)*, respectively. Generally the positive correlation between the accumulation of transcripts of these genes and the increase of freezing tolerance suggests that their products play a role in this phenomenon (Tadeusz, 1998; Vagujfalvi *et al.*, 1999; Motoaki et al., 2001). However the exact function of many cold stress induced proteins have not yet been elucidated.

Like plants freezing tolerant plants, osmotic tolerant plants increase their tolerance to osmotic stress by accumulating molecules and compounds that are thought to function in protecting their cells from stress induced damage. Among these plants *Xerophyta viscosa* Baker has been well described (Sherwin and Farrant, 1996; Mundree and Farrant, 2000). In this regard it was found that *X. viscosa* could survive severe dehydration down to 5% relative water content (RWC). Further, the survival is associated with increased levels of sucrose, sorbitol, polyphenolics, anthocyanins as well as the activity of antioxydant enzymes such as superoxide dismutase, glutathione reductase, ascorbate peroxidase and peroxiredoxins (Mundree and Farrant, 2000).

In order to isolate and characterise *X. viscosa* cDNA genes with roles in osmotic stress, a strategy called " complementation by functional sufficiency" was employed

(Mundree and Farrant, 2000). Screenings permitted the isolation of 9 cDNA clones. One of these shares sequence identity with a cold-regulated gene and was therefore designated *Xvcor*. The *Xvcor* cDNA was 944 bp long and comprised an open reading frame of 792 bp in length, which encodes a extremely hydrophobic protein with six transmembrane domains and has a predicted molecular mass of 29.6 kDa. The amino acids sequence shows 38 % identity to the wheat cold-regulated gene *WCOR413*. Further searches revealed that *Xvcor* lacked signals for organelle targeting suggesting that it might be localised in the plasma membrane.

Despite these data there has not been experimental evidence to elucidate the exact function of the *XVCOR* protein.

To determine the limiting steps to cold tolerance and gain insights into the role of this gene, Xvcor was cloned into a yeast expression vector. The strategy used was based on the constitutive expression of *Xvcor* in yeast. In this report we show that the heterologous expression of the *X*VCOR protein was proved difficult.

Materials and methods

Yeast strain, plasmid and media

Saccaromyces cerevisiae strain W303 (MATα; ade2; his3; leu2; trp1; ura3) was used as the host in this study. The yeast shuttle vector pHVX2 was used as the expression vector . The presence of ampicillin and leucine genes facilitated the selection of *Escherichia coli* and yeast transformants on solid Luria Broth (LB) and Standard Defined (SD) media lacking ampicillin and leucine, respectively.

The yeast strain was grown in sterile YPD (1 % yeast extract, 2 % peptone and 2 % glucose) or in standard defined media (0.67 % yeast nitrogen base without amino acids, 2 % glucose, 20 mg/l of the required amino acids) at 30 °C with shaking. For salt and osmotic stress assays, YPD medium was supplemented with NaCl to a final concentration of 1.2 M and with sorbitol to a final concentration of 2 M, respectively (Lee *et al.*, 1996).

Cloning

The cDNA, *Xvcor* was inserted into the *Eco*RI/ *Xho*I sites of pHVX2, under the control of the phospohglycerate kinase promoter (PGK). The resulting recombinant plasmid was termed pHVXvcor (Figure 4). The construct generated was supposed to drive constitutive expression of *Xvcor* in yeast.

For the ligation, 380 ng and 500 ng of pSK-*Xvcor* and pHVX2 vector respectively were double digested with *Eco*RI and *Xho*I for 3 hours. The digested products were gel-purified on 0.8 % agarose gel by electrophoresis (Sambrook *et al.*, 1989). The bands of interest, 944 bp (insert) and 7513 bp (vector) were then excised from the gel under long UV wavelength exposure. The digested DNA fragments were then extracted using the Qiaex II gel extraction kit (Qiagen). Thereafter, 100 ng of vector and 20 ng of insert (representing a ratio to vector: insert to 1:3) were mixed with 0.5 units of DNA ligase and 2 ul 10X ligase buffer to carry out the ligation for 16 hours. Competent *E. coli* cells JM 109 were then transformed with the ligation mix according to Sambrook *et al.* (1989) and grown on LB media supplemented with ampicillin (50 ug/ml). A Plasmid midi-prep isolation kit (Promega) was used to isolate the putative recombinant plasmid. Restriction enzyme digestion and PCR were employed to test for the insertion and the correct orientation of the insert (Figure 5). The recombinant plasmid was initially amplified in

E.coli cells JM 109, extracted once again and then used to transform competent W303 cells.

Yeast transformation

The recombinant plasmid, pHVXvcor and the vector, pHVX2 (control) were prepared and transferred into competent W303 cells by electroporation. To perform the electroporation, 40 ul of yeast suspension with 5 ul (5 ug) of DNA were mixed in a prechilled electroporation cuvette (0.2 cm) and tapped to the bottom. The electroporation was carried out as recommended by the manufacturers (BIO-RAD, N.Y). Transformants were screened on selective media (lacking leucine).

DNA extraction and PCR procedures

Total DNA from transformed yeast cells was isolated according to Alison *et al.* (1997) and quantified spectrometrically. Samples of 1ug yeast plasmid DNA, 2.5 Units *Taq* DNA Polymerase (Promega), 200 uM dNTPs, and 50 pmol of forward and reverse gene-specific primers 5'-GCACGAGGCAGATTTGAATTG-3' and 5'-ATATGGACACGGATGACCCA-3' respectively, were mixed to perform PCR. The DNA insert was amplified after 30 cycles at 94 °C for 30s; at 61 °C for 45s; at 72 °C for 30s with a final extension step at 72 °C for 6 min.

Stress tolerance assays in yeast carrying Xvcor.

Yeast cells were grown at 30 °C in YPD liquid media for 16 hours. The cell density OD_{600} was then checked and adjusted to 2. In order to determine the difference in growth, 1:10 serial dilutions were made. Then, 10 ul of each dilution was spotted on YPD solid media

and incubated at 30 °C (control, salt stress, and osmotic stress) at 4 °C (cold stress) and at 42 °C (heat stress) for 3 days.

Extraction of yeast plasma membrane proteins

The process was based on the sucrose gradient separation protocol (Serrano, 1988) modified by Tsekoa (2001). Yeast cells grown in SD liquid media were collected by centrifugation at 4 °C and washed twice with ice-cold water. The resulting cells were then resuspended in ice-cold homogenising buffer (100 mM Tris-HCl, 50 mM NaCl, 0.1mM PMSF). The suspension was Ball-milled in a Braun Mill using glass beads. Insoluble material was removed by spinning the sample at 700g for 10min at 4 °C. The supernatant representing total proteins (determined by Bradford method) was transferred to fresh tubes and centrifuged for 20 min at 20 000g at 4 °C. The supernatant was then poured off and the pellet, enriched with plasma membranes, retained and then resuspended in 20 % glycerol. To separate the membrane mixture, the resuspended pellet was transferred to ultracentrifugation tubes, containing a discontinuous sucrose gradient composed of 8 ml 53 % sucrose at the bottom and 16 ml 43 % at the top. Centrifugation was then carried out at 25 000 rpm for 6 hours at 4 °C. The purified plasma membrane band was found at the interface between 53 % and 43 % sucrose layers. This band was collected using a Pasteur pipette, diluted in water and centrifuged for another 20 min at 80 000g at 4 °C. The pellet was resuspended in protein sample application buffer and used for SDS-PAGE analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to Laemilli *et al.* (1970) with some modifications (Harlow and Lane, 1980). Throughout this study, 12 % separating gel and 6 % stacking gel were employed at 200V constant voltage for 7 hours to resolve proteins.

Total RNA extraction and RT-PCR

Yeast total RNA was extracted according to Sherman *et al.* (1986). Then sample of 1ug/ul total RNA was added to 1uM oligo$(dT)_{15}$, 10 units RNAse inhibitor and 4 units of M-MuLV Omniscript reverse transcriptase to perform the reverse transcription in a 20 ul total volume. The manufacturer's protocol (Qiagen) was followed. PCR was then performed as described in material and method (DNA extraction and PCR procedures).

Results

Sequence analysis of Xvcor cDNA

The *Xvcor* ORF is 792 bp in length and encodes a highly hydrophobic protein, which comprises 264 amino acid residues (Figure 1), contains six transmembrane domains (Figure 1 and 3), and has a predicted mass of 29.6 kDa. No particular conserved motif has been identified within the amino acid sequence. A computer search for similarities using BLAST revealed high degree identities to several putative cold acclimation proteins. The best identities scores were to *Arabidopsis thaliana (*44.26 %); *Triticum aestivum* (41.89 %); *Oryza sativa* (43.24 %) (Figure 2). Interestingly, data documented to date failed to reveal a homologous protein in yeast.

Cloning Xvcor into the yeast expression vector

The presence of *Xvcor* in yeast was verified by PCR. Gene specific primers were used to perform PCR on total DNA extracted from W303 cells, W303 transformed with the vector alone, and W303 transformed with the recombinant plasmid. The predicted band product (944 bp) was specific to W303 cells transformed with the recombinant plasmid. This confirmed that *Xvcor* was successfully cloned in yeast (Figure 6).

Testing for heterologous expression of Xvcor in S. cerevisisae

A key factor determining the amount of a particular protein in the cell is the accumulation of the corresponding transcripts. RT-PCR with gene-specific primers and oligo$(dT)_{15}$ (or reverse primer) was performed on total RNA extracted from recombinant W303 cells. A prominent DNA fragment of the expected size (944 bp) was identified only for W303 cells transformed with the recombinant plasmid. Additionally, PCR performed on total DNA extracted from the cells used earlier (Figure 7) amplified an identical DNA fragment. This result indicated that *Xvcor* was transcribed in the yeast cells transformed with the recombinant plasmid.

To determine if the XVCOR protein accumulated in yeast, total and plasma membrane proteins were extracted from the yeast cells transformed with the empty vector (control) and the recombinant plasmid as described in Materials and Methods. Both samples were then electrophoresed on SDS-PAGE and the proteins analysed by staining the gel with Coomassie brilliant blue (Figure 8). Surprisingly, we could not detect any difference between the positive recombinant and the control; suggesting that either the level of the heterologous protein was extremely low or that there was no recombinant protein at all. In addition it should be considered that hydrophobic proteins are difficult to isolate.

Despite this, yeast cells were exposed to various stress conditions, to check whether little amount of recombinant protein (undetectable by SDS-PAGE analysis) was produced, and could alter stress tolerance in transgenic yeast.

Stress tolerance assays performed on Xvcor transformants

To test if the presence of *Xvcor* enhanced osmotic, salt, heat and cold stress tolerance (despite the fact that no stress protein was detected by SDS-PAGE), yeast cells were grown in SD liquid medium overnight. The cultures were then diluted and spotted on solid YPD medium (control) and on YPD, which was supplemented with sorbitol (osmotic stress), and with NaCl (salt stress). Cold stress and heat stresses were achieved by incubation at 4 °C and 42 °C, respectively for 3 days. There was no difference in the growth performance of transformed and untransformed cells. Furthermore the transgenic strains evolved identically and displayed the similar phenotype to the wild type (Figure 9). This finding confirmed that attempts to express *Xvcor* had not led to a stress protein or the protein did not confer tolerance. Identical results were found in experiments performed in YPD liquid media as well as on SD solid media (data not shown).

Taken together these results indicated that *Xvcor* was efficiently transcribed in yeast, but the absence of heterologous protein implies that translation was hampered.

Codon adaptation index (CAI) of Xvcor in yeast

To predict and optimise the expression level of heterologous proteins, Sharp and Li (1987) designed an algorithm termed codon adaptation index with an expression scale from 0 to 1. In order to explain the lack of expression in yeast, we did codon usage analysis. It revealed that codon adaptation index of *Xvcor* in yeast is 0.11, confirming that expression of *Xvcor* in yeast is extremely low and thus potentially problematic.

Considering that XVCOR protein plays a role in stress tolerance in *X. viscosa*, we reasoned that overexpression of XVCOR protein in a different organism might certainly

provide insights regarding its function. The approach used in this study utilized a yeast expression vector and yeast cells W303 as heterologous host. The gene was placed under the control of a strong promoter (PGK). This was supposed to achieve constitutive expression of the recombinant protein in yeast. Since the expression *Xvcor* did not succeed, we cannot assign a particular biological function to the XVCOR protein.

Discussion

Yeast is a good model system for biological studies. Yeast not only possesses the ease of handling of prokaryotes cells, they also exhibit the qualities of eukaryotes (Bill, 2001). Furthermore, yeast is known to survive various types of stresses and many investigations based on the complementation analysis have underlined the role and function of stresses inducible genes in yeast (Lee, 1999; Tsekoa, 2001; Nyander *et al*, 2001). To determine the role of *Xvcor*, we cloned the cDNA into yeast expression vector and transformed *S. cerevisiae*. We examined its effects on growth tolerance under salt, osmotic, cold and heat stresses.

Despite the fact that *Xvcor* was transcribed, no heterologous protein was found on SDS-gels. Furthermore the recombinant cells and the control displayed identical phenotype and similar growth performance under osmotic, salt, heat and cold stresses. The absence of a phenotype imparted on the transformed yeast presumed a failure to express *Xvcor* in yeast

In contrast to our failure to express *Xvcor* in yeast, Mundree and Farrant (2000) cloned *Xvcor* in *E.coli* cells and selected the transformants for their ability to tolerate an osmotic pressure. This result, along with the fact that investigations failed to express XVCOR protein in yeast, implies problems with yeast as host. Effectively, bioinformatics

searches revealed that the codon usage bias in *Xvcor* was extremely low, compared with that of the highly expressed genes in *S. cerevisiae*. For example the *S. cerevisiae* LEA-like protein HSP12, which is highly expressed at the plasma membrane level under stresses, had a codon adaptation index corresponding to 0.639 (Sales *et al.*, 2000). Recent investigation found that HSP30 that was moderately overexpressed in yeast under ethanol stress has a codon adaptation index corresponding to 0.230 (Tsekoa, 2001). In our case, these data suggest that the codon usage in *Xvcor* negatively influenced the expression of the heterologous protein in yeast. Therefore we can assume that there is a positive correlation between the codon usage variation and the level of expression of genes in *S. cerevisiae*. Investigations in yeast (Chiapello *et al.,* 1999) had already converged to an identical remark. However this reason is not totally convincing since the codon usage of the same gene in *E.coli* was found to be 0.187. In fact, it is easier to identify heterologous protein in *E.coli* than in yeast; however these proteins are more often misfolded and inactive. In yeast, the expression also depends of post-translational modifications that lead to the correlation between the structure and the function.

The RT-PCR results as well indicated that *Xvcor* is transcribed. Since *Xvcor* was placed under the control of a strong promoter (PGK), and the plasmid expression vector engineered to contain an origin of replication controlling a high copy number, it is possible that the failure of expressing the heterologous protein in yeast is due to a defect at the translational level.

However, it is also possible that the absence of heterologous protein on SDS-gels be due to the absence of detectable amount of XVCOR protein. This is in accord with the fact that membrane proteins are highly hydrophobic and are therefore difficult to reveal in such

conditions. This is questionable since both the recombinant and the control displayed similar growth performance and phenotype under stresses. Therefore it seems more likely that the absence of heterologous protein was due to a defect at the translational level. Similarly, the nature of the media and the yeast growth phase should be considered. In this order, investigations showed that the expression of plant group 2 and group 3 *lea* genes in yeast was significantly enhanced during the lag phase in liquid media, whereas no difference was found in their growth performance on solid media (Zhang *et al.,* 2000*)*.

More specifically, recent investigations in the field of membrane proteins indicated that they are generally poorly overexpressed. The reason for this is not fully understood; however it is suggested that the post-translational modifications that are necessary to attain their 3D conformation; in addition with the pathways, accessory proteins and chaperones that involve in this process, are still unknown. In yeast for example, studies demonstrated that the key element distinguishing their post-translational modifications from those of other eukaryotes is abundance of mannose residues in their oligosaccharide chains (Bill *et al.,* 1998). This hypermannosylation is believed to affect heterologous protein structure and function (Bill, 2001). Another factor that could influence the heterologous expression of membrane proteins is its interaction with its native protein and other proteins in its homologous host (Bill, 2001). This suggests that in a heterologous system, non-specific interactions may result in a different function and structure.

In summary the results presented here show that *Xvcor* is efficiently transcribed in yeast. However, constraints related to the host hamper its translation. As a consequence, we cannot assign a function to this putative cold acclimation protein. However, the fact that the predicted protein is extremely hydrophobic along with its high size suggest that it might be

indeed an integral part of the plasma membrane and could perform various functions such as ion transport or provide receptor sites for extracellular signals. In the short term, future work will aim to use an approach based on the inducible expression system instead of a constitutive one, and quantitative RT-PCR, to measure the amount of mRNA accumulated in the transgenic yeast under stress and normal conditions. In the long term, studies must take advantage of bioinformatics tools and the fact that the whole yeast genome is sequenced. This makes possible to screen within *Xvcor* sequence and identify the least used codons in yeast. Changing specific sequences by site-direct mutagenesis could solve the problem of codon bias.

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CGG CAC GAG GCA GAT TTG AAT TGA ATT TGC TGC GTT TTG GTT TTG GCA GAT TTG
AGC GAG GGA GAT TAG GGA AG                                          -75

 M   R   N   E   G   F   L   K   M   K   T   D   V   G   V   A   D   E    18
ATG AGG AAC GAG GGT TTT CTG AAA ATG AAG ACC GAC GTT GGA GTC GCC GAC GAG    54

 V   I   S   G   D   L   K   Q   L   G   D   A   A   K   R   L   A   K    36
GTG ATC TCC GGA GAT CTC AAG CAG CTT GGT GAC GCT GCA AAG CGG CTA GCT AAA   108

 H   A   I   K   L   G   A   S   F   G   V   G   S   T   I   V   Q   A    54
CAT GCG ATC AAG CTC GGC GCC AGC TTC GGG GTT GGC TCT ACC ATA GTC CAG GCT   162

 I   A   S   I   A   A   I   Y   L   L   I   L   D   R   T   N   W   R    72
ATT GCT TCG ATC GCT GCT ATC TAT TTG TTG ATA TTG GAC CGG ACA AAC TGG CGT   216

 T   N   I   L   T   S   L   L   I   P   Y   V   Y   L   S   L   P   S    90
ACA AAT ATC TTG ACA TCA CTT CTA ATT CCA TAT GTT TAC TTG AGT CTT CCT TCA   270

 V   I   F   N   L   F   R   G   D   L   G   R   W   L   S   F   I   G   108
GTG ATA TTC AAC CTA TTC AGG GGC GAC CTG GGC AGA TGG CTT TCA TTC ATT GGC   324

 V   V   M   K   L   F   F   H   R   H   F   P   V   T   L   E   L   L   126
GTA GTA ATG AAG CTC TTC TTC CAC CGA CAC TTC CCA GTT ACC TTG GAA CTG CTT   378

 V   S   L   I   L   L   I   V   V   S   P   T   F   I   A   H   T   I   144
GTG TCT CTC ATT CTC CTG ATT GTG GTT TCC CCC ACT TTC ATT GCC CAC ACA ATC   432

 R   G   S   L   I   G   V   F   I   F   L   V   I   A   C   Y   L   L   162
AGA GGC AGT CTC ATT GGA GTC TTC ATC TTC CTT GTC ATC GCC TGC TAC CTC CTC   486

 Q   E   H   I   R   S   A   G   G   F   K   N   A   F   T   K   S   N   180
CAA GAG CAC ATT AGA TCA GCT GGT GGC TTC AAA AAC GCG TTC ACA AAG AGC AAT   540

 G   I   S   N   S   V   G   I   I   I   L   L   I   H   P   I   W   S   198
GGG ATT TCA AAC AGC GTC GGG ATC ATC ATT CTA CTG ATC CAC CCG ATC TGG AGC   594

 L   V   V   Y   F   L   Y   T   S   L   L   Q   L   L   A   Y   S   P   216
TTG GTG GTG TAT TTC CTC TAC ACG TCT TTG CTG CAA CTT CTT GCA TAC TCT CCT   648

 S   P   C   C   C   I   L   Y   N   K   W   F   N   F   M   H   V   C   234
TCC CCT TGT TGT TGC ATA TTA TAC AAT AAG TGG TTT AAT TTC ATG CAT GTT TGT   702

 K   C   V   S   L   H   M   Y   S   Q   S   I   G   S   C   V   S   I   252
AAA TGT GTA AGC CTT CAT ATG TAT TCT CAG TCA ATT GGG TCA TGC GTG TCC ATA   756

 F   F   V   Q   F   V   F   I   Y   E   A   E   F   *                   266
TTT TTC GTG CAG TTT GTA TTC ATC TAT GAA GCT GAA TTT TAA                   798

GAT TCA TGT ATA TGC TCT TTG GTA GAT ATG TGG AGG TAC CGT TGT TCT TTA AAA AAA  930
AAA AAA AAA AAA AAA                                                      944
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Figure 1.Nucleotide sequence and deduced amino acid sequence of *Xvcor*. The putative start and stop codon are represented in bold. The transmembrane domains are underlined. (Garwe *et al* 2002)

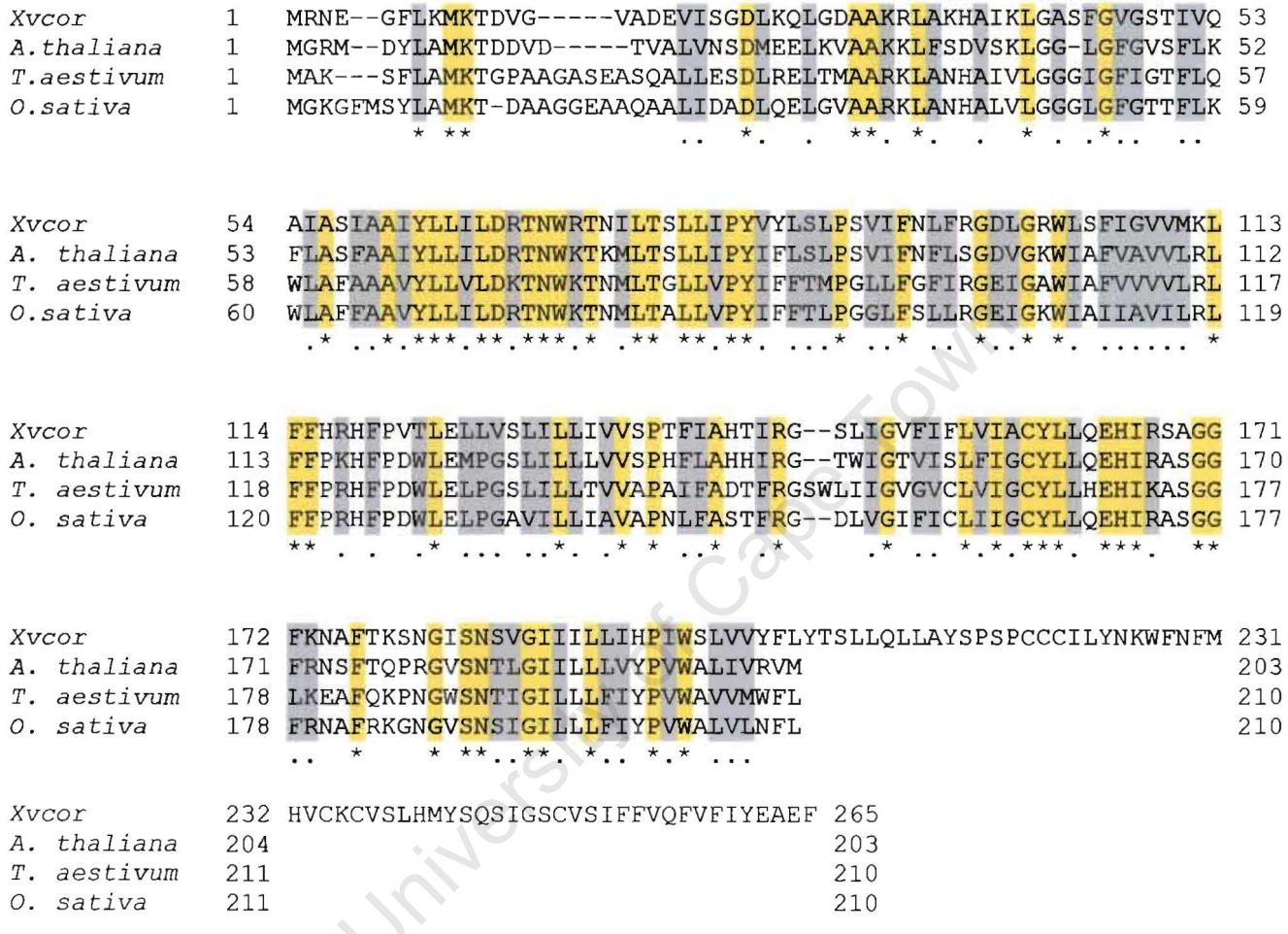

Figure 2.Amino acid comparaison of Xvcor with related proteins.
Asterices(*) indicate identity; dots(.) indicate similarity. (Garwe *et al* 2002)

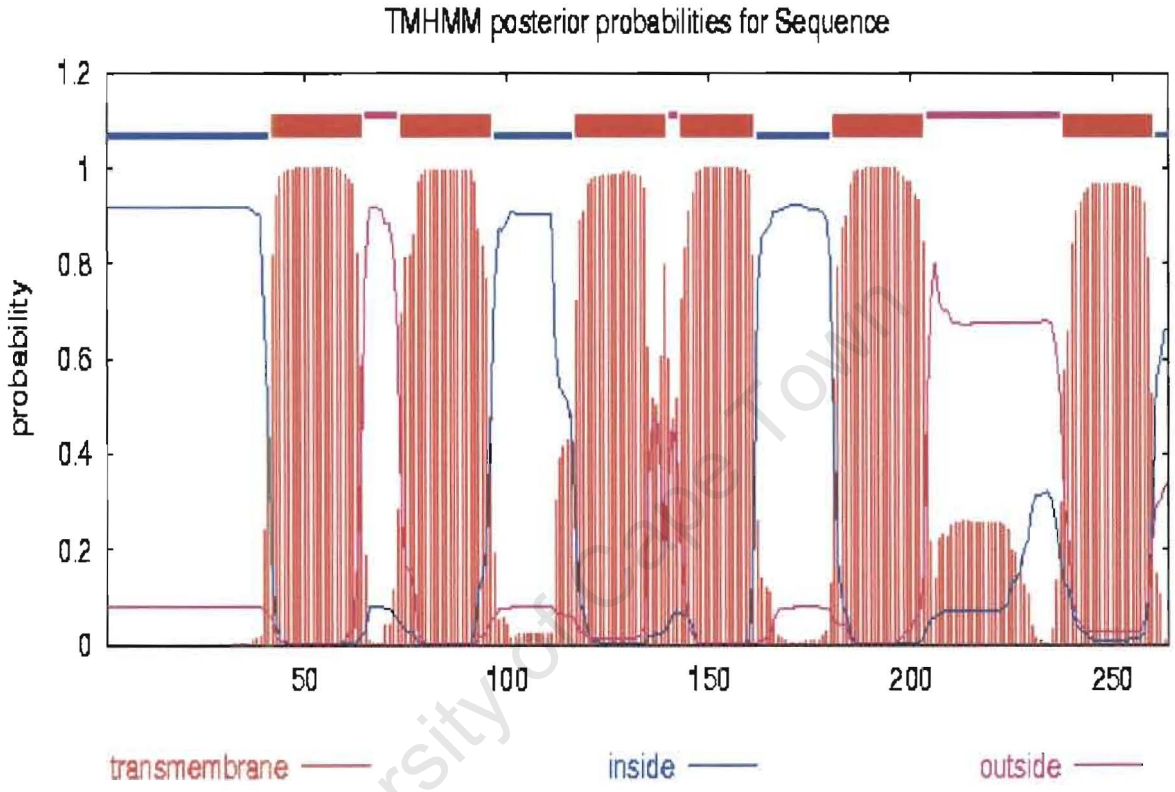

Figure 3. Transmembrane domains of the Xvcor deduced amino acid sequence using 50-amino acids window (Garwe *et al* 2002)

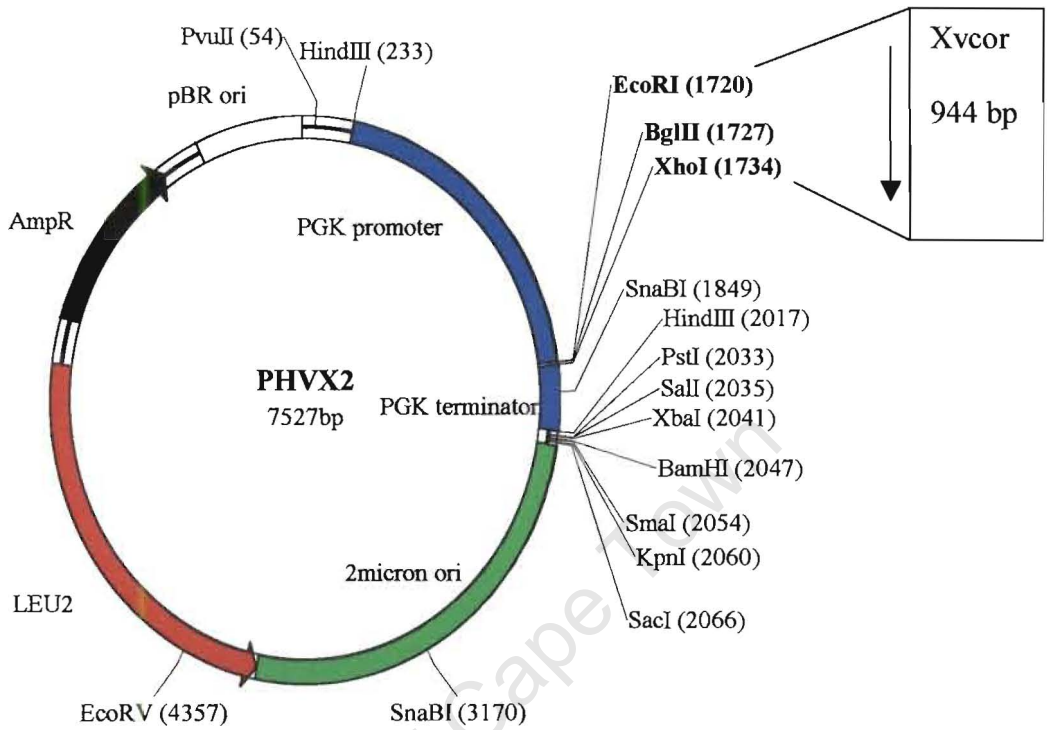

Figure 4.The plasmid vector pHVX2 (Tsekoa, 2001) used to express *XVCOR* in *S. cerevisiae*. The gene was cloned into the *Eco*RI and *Xho*I restriction sites.

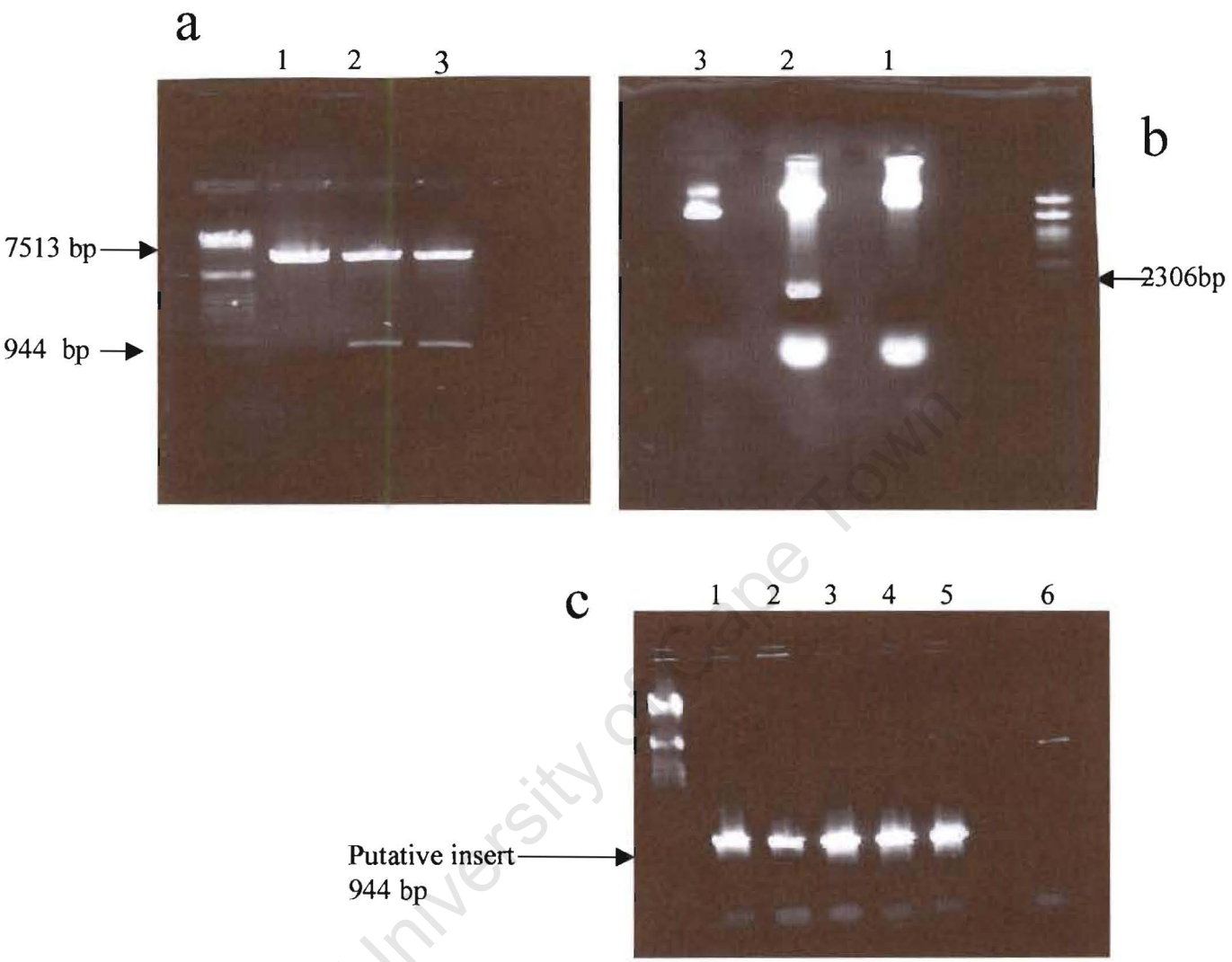

Figure 5. Tests for insertion. The putative recombinant plasmid was extracted from *E. coli* cells. Restriction enzymes digested (a and b) and PCR (c) were employed to test for the insertion and the orientation. Double digestion using *EcoR*I and *Xho*I (a) revealed a DNA fragment with the expected size (2 and 3) whereas the 13 bp DNA fragment expected from the empty vector is not observed. Double digestion (b) using *Bamh*I (specific to the vector) and *XbaI* (Cut within Xvcor) revealed the expected DNA fragment of 2306 bp (2) and confirmed the sense orientation of the insert. Lanes 1 and 3 represented the recombinant and the empty plasmid respectively. PCR performed with increasing concentrations of DNA (1, 2, 3, 4, 5); the vector alone (6) was used as negative control. Lambda *Pst* was loaded as marker.

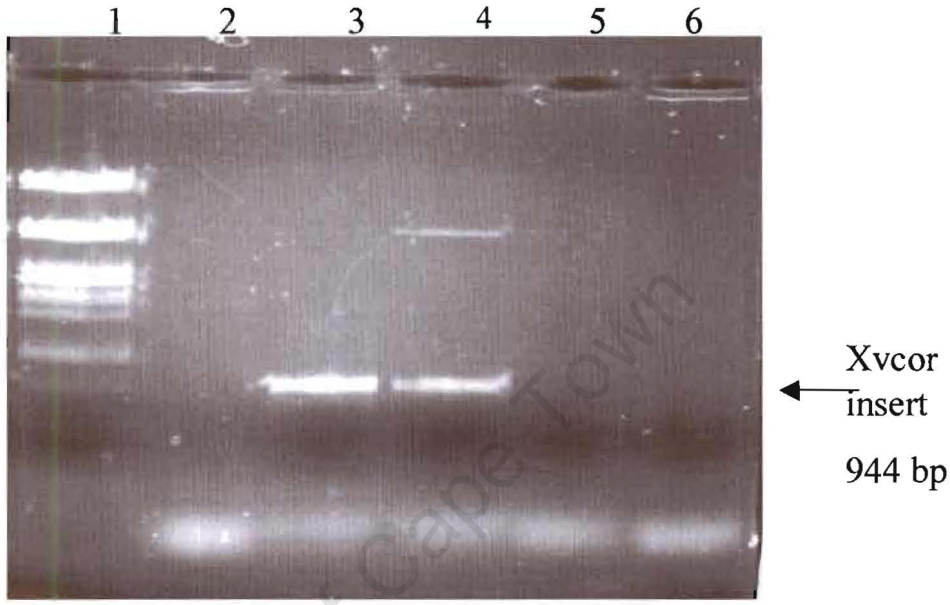

Figure 6. The presence of *Xvcor* in yeast cells was verified by PCR using gene specific primers. Yeast cells were grown overnight at

30 °C. Total DNA was extracted from W303 cells (2), W303 cells transformed with the recombinant plasmid (3) and with the vector alone (6) and amplified. The recombinant plasmid was used as positive control (4). Lane 5 was loaded with PCR products. Lambda DNA was employed (1) as marker.

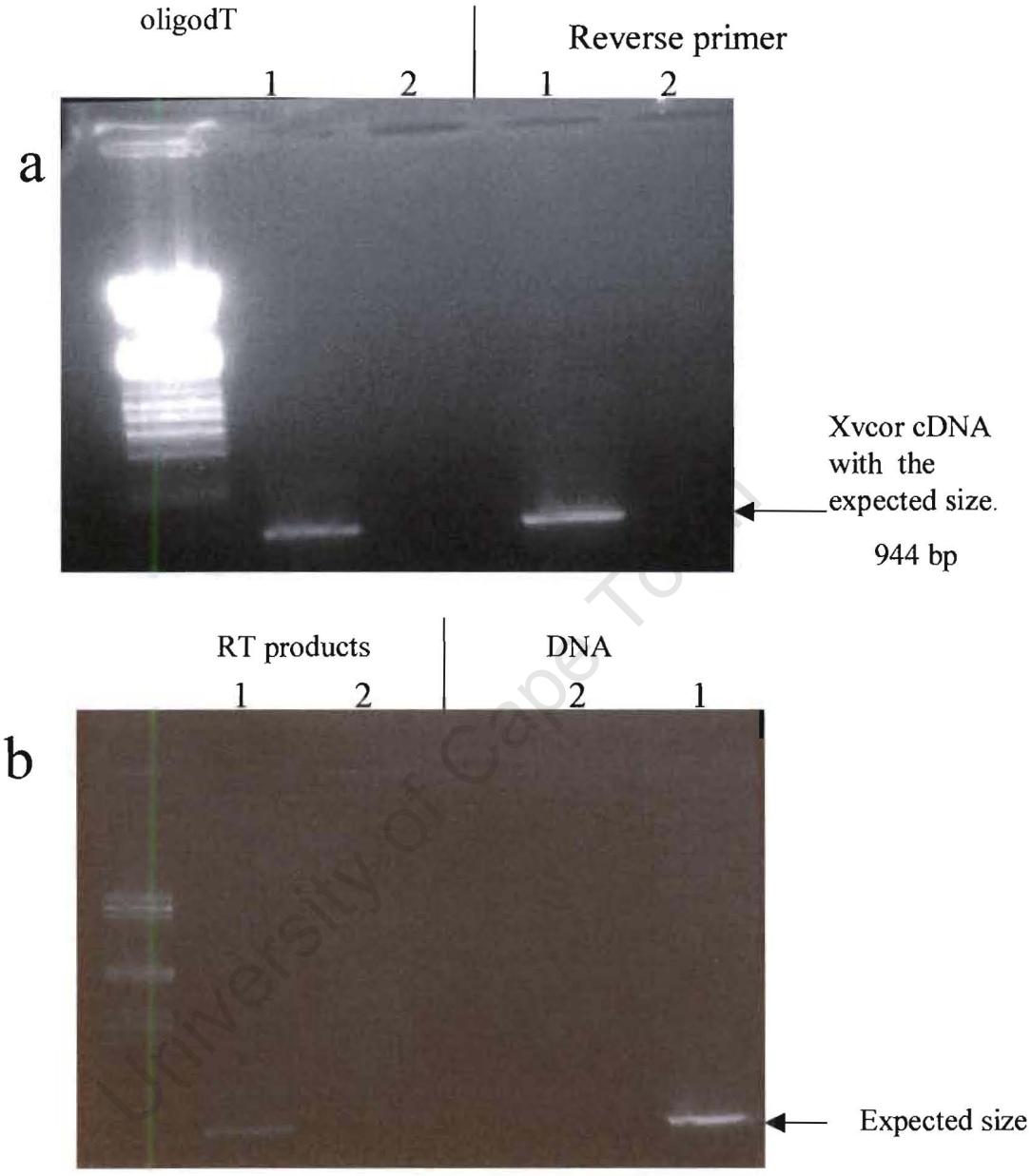

Figure 7. Transcripts accumulation in yeast. RT-PCR (a) was performed on total RNA, using oligodT and reverse primer. Transformants were grown at 30 °C overnight in SD liquid media.1 ug/ul total RNA extracted from W303 cells transformed with recombinant plasmid (1) and from W303 cells transformed with pHVX2 alone (2) were used. PCR (b) was performed to compare DNA extracted and RT-products.

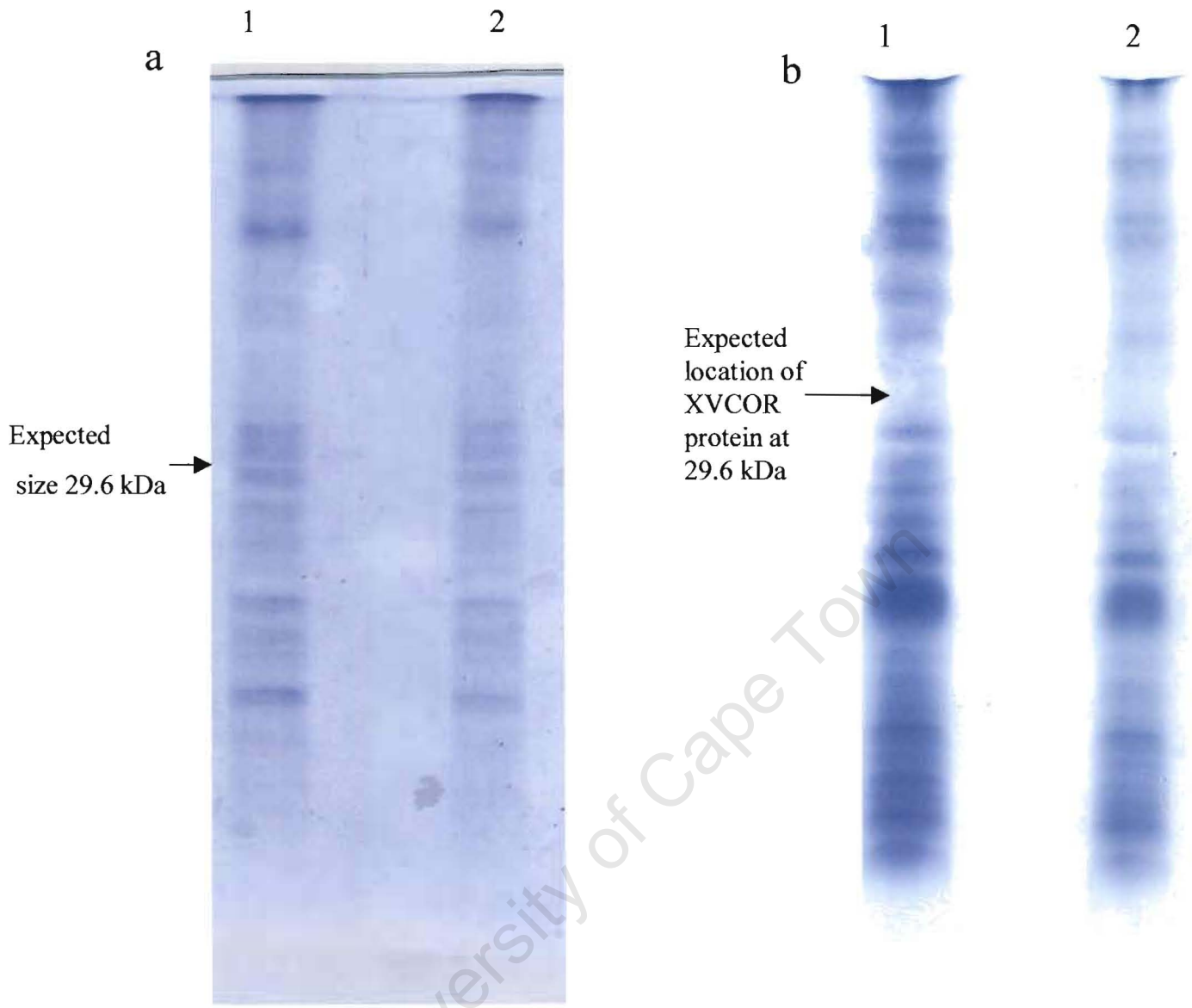

Figure 8. Test for Xvcor protein. Total (a) and plasma membranes (b) were extracted from W303 transformed with PHVXCOR (lane1) and from W303 transformed with PHXV2 (lane 2). Proteins were separated by SDS-PAGE and stained with Coomassie Brillant Blue. No difference difference was found.

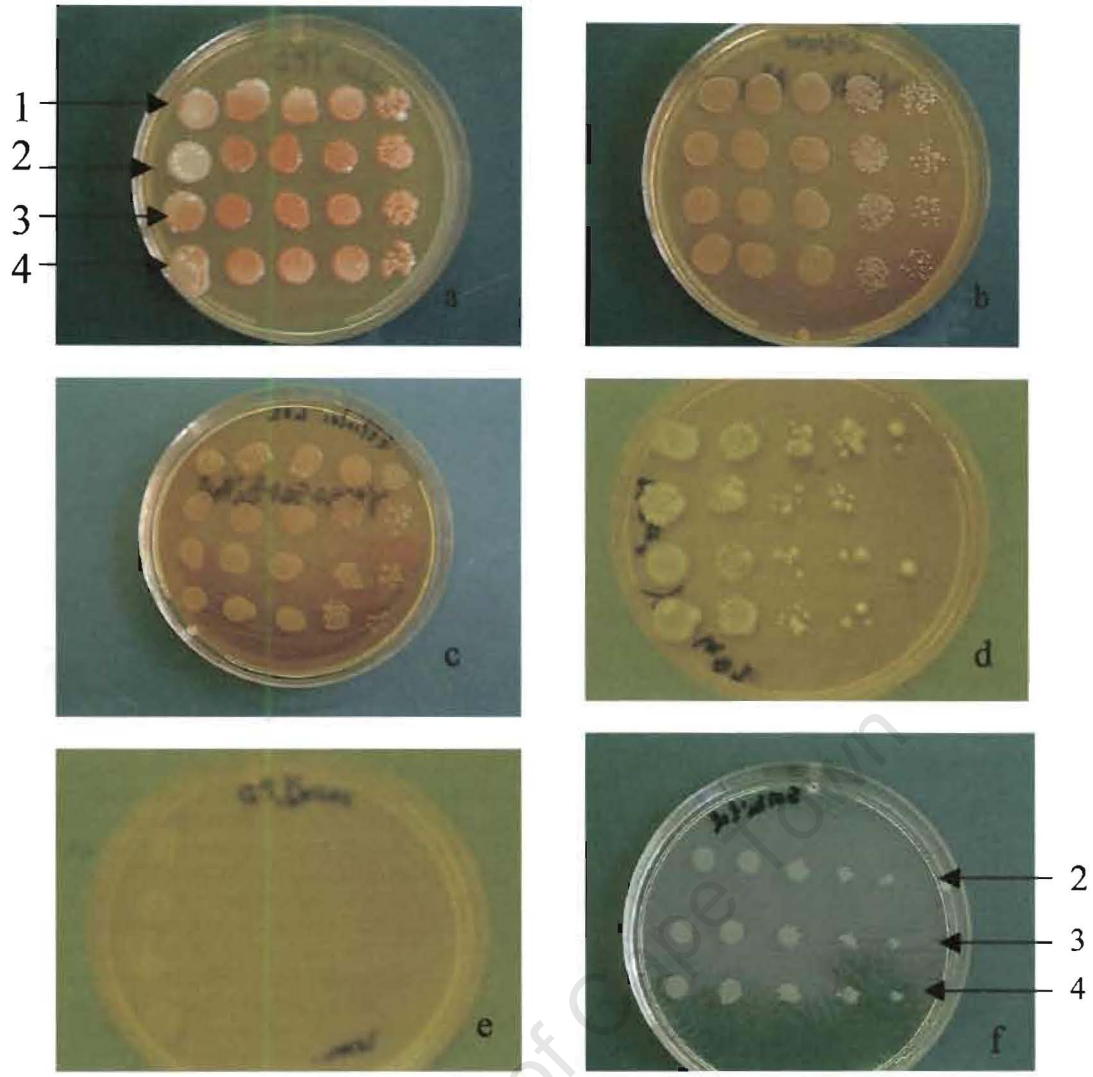

Figure 9 . Stress tolerance assays in yeast carrying *Xvcor* . YPD solid medium (a) was used as control. It was then supplemented with 1.2 M NaCl for salt stress (b), 2 M sorbitol for osmotic stress (c). For heat stress (d) and cold stress (e), plates were exposed at 42 °C and 4 °C, respectively for 3 days. Similar results was found on SD media as shown for osmotic stress (f). 1:10 serial dilutions (columns) were made at each step, 10 ul spotted on solid media. Shown are plates incubated for 5 days. W303 cells (lane 1); W303 + recombinant vector (lane 2); W303 + vector pHVX2 alone (lane 3); W303 + recombinant vector (lane 4).

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